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GRANT NO:

DAMD17-93-J-3006

TITLE:

Markers of Fibroblast Growth Factor Family-Mediated Growth Signal Transduction as Determinants of Successful Hormonal Therapy for Patients with Estrogen Receptor Positive Breast Cancer

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REPORT DATE:

May 16, 1995

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

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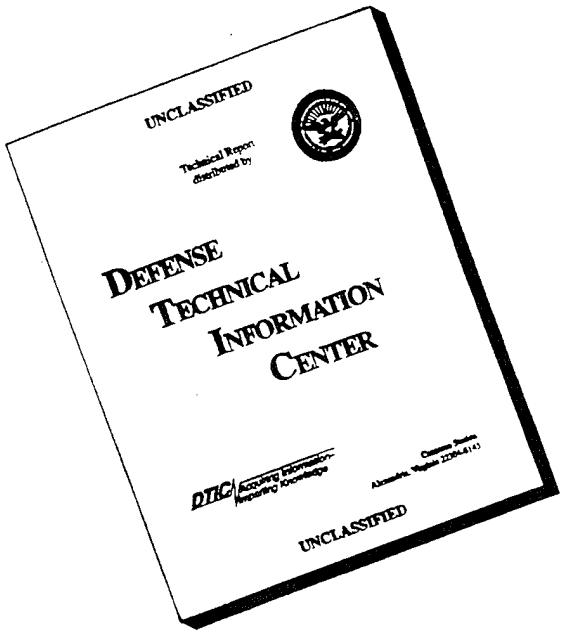

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OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 16, 1995	Final: 12/15/92 - 12/14/94

4. TITLE AND SUBTITLE
Markers of Fibroblast Growth Factor Family-Mediated Growth Signal Transduction as Determinants of Successful Hormonal Therapy for Patients with Estrogen Receptor Positive Breast Cancer

5. FUNDING NUMBERS
DAMD17-93-J-3006

6. AUTHOR(S)
Francis G. Kern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University

Washington DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release,
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

To determine whether FGFR-3 expression correlated with antiestrogen resistance, two high titer polyclonal rabbit antibodies were generated against two peptides present in the FGFR-3 protein. Pathological specimens from 181 patients with estrogen receptor positive breast tumors that received tamoxifen therapy were identified. H+E stained sections were evaluated and tumor tissue sections were prepared from paraffin embedded material from over 100 patients. To determine the suitability of the FGFR-3 antibodies for use in immunohistochemical assays with sections from formalin-fixed and paraffin-embedded material, we sought to establish a tumorigenic cell line that overexpressed FGFR-3 for use as a positive control. A cDNA clone purported to contain the complete coding sequence for FGFR-3 was inserted into a eucaryotic expression vector. MCF-7 breast carcinoma cells transfected with this vector were characterized but no protein expression was observed due to an unexpected insertion of DNA into the coding region of the cDNA. Reverse transcription- polymerase chain reaction (RT-PCR) was used to generate plasmid transcription vectors for FGF-7 and a FGFR-2 splice variant that generates a specific receptor for FGF-7. These vectors were used to prepare probes for RNAse protection assays for FGF-7 and its receptor. The presence of FGF-7 RNA was detected in 35 of 36 human breast tumors examined. The probe for the FGFR-2 splice variant was not suitable for the RNAse protection assay. A semi-quantitative RT-PCR assay that was developed as an alternative showed expression of the FGF-7 specific receptor in six of nine tumors examined.

14. SUBJECT TERMS
breast cancer, antiestrogen resistance, fibroblast growth factors, growth factor receptors

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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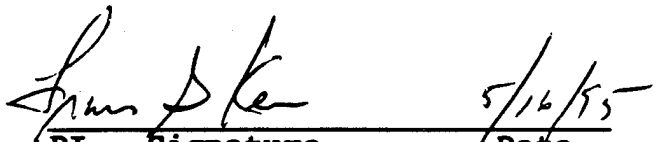

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INTRODUCTION:

A response to tamoxifen therapy is observed in less than 60% of breast cancer patients with recurrent disease that initially presented with estrogen receptor positive tumors. Therefore additional markers are needed that will allow the clinician to better predict that subpopulation of patients most likely to respond to this therapy. Our laboratory has demonstrated that overexpression either FGF-1 or FGF-4, which are two members of the fibroblast growth factor family, in an estrogen receptor positive breast cancer cell line confers these cells with the ability to grow tumors in ovariectomized athymic nude mice treated with tamoxifen (1,2). These results suggest that FGF signal transduction mediated through FGF binding to specific transmembrane tyrosine kinase receptors may be capable of overcoming the estrogen dependence of estrogen receptor positive (ER+) breast cancers for growth. Indicators of FGF signal transduction being operative within an estrogen receptor positive tumor might therefore be capable of providing a sign of the likelihood that tamoxifen therapy will be ineffective. These indicators could include evidence of expression of either FGF ligands or FGF receptors. Nine members of the FGF family of ligands have been identified to date and four transmembrane tyrosine kinase receptors activated by ligand binding have also been described (3-6). We have used sensitive and specific RNAse protection assays to examine the range of expression of six of the nine FGF ligands and all four receptors in human breast tumors. With such an assay we have found that FGFR-3 is highly expressed in a subset of tumors (7). One objective of this project included raising antibodies that could be used in an immunohistochemical assay for FGFR-3 expression. Such antibodies would then be used to perform a retrospective study on formalin-fixed and paraffin-embedded material from patients with ER+ tumors that received tamoxifen treatment and for whom follow up information could be retrieved. This would allow one to determine if FGFR-3 overexpression correlates with the failure of tamoxifen therapy. A second objective was to develop RNA probes that could be used to examine the range of expression of FGF-7 expression and its receptor in human breast cancers.

BODY:

A. Preparation of polyclonal rabbit antisera against FGFR-3

1. Choice of peptide antigens.

Two hydrophilic, extracellular portions of FGFR3 were chosen as potential antigens using the PlotStructure and Helicalwheel programs in the GCG software package, and using the Antigen program in the PCGENE software package. They are (1) peptide-a: RAAEVPGPEPGQQEQ, corresponding to amino acids 33-48 (15-mer) of FGFR-3 and (2) peptide-b: PQRLQVLNASMEDS, corresponding to amino acids 91-105 (14-mer) of FGFR-3. These two hydrophilic and extracellular portions where also chosen based on their limited homology to corresponding regions in FGFR-1, FGFR-2 and FGFR-4. 50 mg of HPLC purified peptide-a and peptide-b were synthesed by BioServ Labs (San Jose, CA).

2. Peptide coupling and rabbit immunization.

An additional cysteine residue was attached to the carboxy terminus of both peptides during synthesis to facilitate coupling to carrier. 25 mg of peptide-a and peptide-b were conjugated to keyhole limpet hemocyanin (KLH) using the heterobifunctional reagent, m-malemidobenzoyl-N-hydroxysuccinimide (MBS). For each KLH-coupled peptide, two New Zealand white rabbits, female, weight 3 kg, were immunized by subcutaneous injections into multiple sites on the rabbit's back. The first immunization was performed with 0.5 mg of peptide-KLH and subsequent boosts were performed with 1mg of peptide-KLH. Complete Freund's adjuvant (GIBCO) was used in the first immunization and incomplete Freund's adjuvant was used in later boosts. The ratio of antigen:adjuvant was 1:1 (v/v). The animals received their first boost three weeks after the primary injection and subequent boosts were continuously carried out at intervals of 14 days. A prebleed was done immediately before the first immunization. Bleeds from the ear vein and artery were carried out 7 days after boosts.

3. Determination of the titer of anti-FGFR3 in immunized rabbit serum.

An enzyme linked immuno absorbant assay (ELISA) for the titer of anti-FGFR-3 was developed as follows. 200 ng/well of peptide-a or peptide-b was added to each well of a 96 well of Reacti-Bind™ maleic anhydride activated polystyrene plates (PIERCE) and incubated at 37°C overnight. After washing, 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) was added at room temperature for 1 hour followed by washing with PBS with 0.1% BSA and 0.05% Tween-20 (wash buffer). The rabbit serum was diluted at 1:6,000; 1:12,000; 1:24,000; 1:48,000 in 0.5% BSA-PBS and 200 ul of the diluted immunized rabbit serum was added to the well to allow the anti-FGFR-3 peptide antibodies to bind to corresponding peptide. After incubation at room temperature for 1 hour, the nonspecific protein in the serum was removed by three washes with wash buffer. 200 ul of 1:2000 diluted horseradish peroxidase (HRP) conjugated sheep anti-rabbit IgG was added to the wells and further incubated for 1 hour at room temperature. After three washes to remove unbound second antibodies, 200 ul of HRP substrate, 3,3',5,5' tetramethyl benzidine (TMB, 0.4g/l, PIERCE) was added to the well to develop the color. The reaction was stopped with 100 ul of 2M H_2SO_4 The plates was read at A_{450} in Auto-ELISA reader. The prebleed serum at the same dilution was used as background control. The results of the ELISA assays of anti-FGFR-3 from 4 rabbits is included in Figure 1 and Figure 2 in the Appendix. They indicate that for all four sera, positive titers were still obtained at the highest dilution tested (1:512,000).

B. Development of an immunohistochemical assay for FGFR-3.

1. Identification of specimens from patients that received hormonal therapy.

Information contained within the Georgetown University Medical Center tumor registry was used to generate a list of 181 patients that received tamoxifen therapy. Pathological specimens from approximately 160 of these patients were available through the Georgetown University Medical Center Pathology Department. The paraffin blocks from each of these patients that contained breast tumor tissue was determined by examining a representative hematoxylin and eosin (H+E) stained section from each block. Microscopic sections from each block shown to contain tumor tissue were prepared from 110 different patients. When metastatic lymph node tissue was also available, a section was also obtained from the block.

2. Construction of an FGFR-3 expression vector and transfection of MCF-7 human breast carcinoma cells.

Not all polyclonal antibodies are suitable for use with formalin-fixed and paraffin embedded material. False positives can result from lack of specificity and false negatives can occur from lack of sensitivity. The latter problem can sometimes be alleviated by unmasking epitopes that may have been destroyed in the fixation process through a process known as antigen retrieval by microwave boiling (8). Frozen tissue was not available from any of the archival tumor tissues used for tissue sectioning We therefore could not use RNAse protection assays to determine the levels of FGFR-3 expression in any of the tumor samples and paraffin blocks were not available for tumor tissues that we had previously used for our survey of FGFR-3 mRNA expression using RNAse protection assays. We therefore had no way of determining either the sensitivity of specificity of the FGFR-3 antibodies we had generated.

In an attempt to bypass this problem, we decided to transfect the MCF-7 human breast carcinoma cell line with an FGFR-3 expression vector. This cell line produces a level of FGFR-3 mRNA that is detectable in an RNAse protection assay only after long exposures of the autoradiograph. Although the cell line can respond to either exogenous or transfected FGFs, levels of receptor expression border on the limits of detection using conventional ^{125}[I]-FGF binding assays (1,2,9) and would therefore not be expected to be detected in an immunohistochemical assay using either of the FGFR-3 antibodies we had generated. The cell line transfected with the empty vector could therefore be used as a negative control to test the specificity of the antibodies and cell lines transfected with the vector containing a full length FGFR-3 cDNA insert expressing different amounts of the protein could be used to test the sensitivity. Since the cell line is tumorigenic in athymic mice receiving estrogen supplementation, the tumors could be snap-frozen or formalin-fixed and paraffin-embedded. This would allow us to determine if the fixation and embedding process was affecting sensitivity. If this were observed,we could then determine if antigen retrieval by microwave boiling could alleviate the problem. The tumors could also be used to establish the proper antibody dilutions and conditions required for sensitive and specific FGFR-3 detection prior to our using the antibody on the archival samples. If the approach proved successful, subsequent transfections of MCF-7 cells with FGFR-1, FGFR-2 and FGFR-4 expression vectors could be used to establish the lack of crossreactivity of the FGFR-3 antibodies we had generated with any of the related family members.

We had obtained form Dr. Kari Alitalo a plasmid containing an 5.0 kb FGFR-3 cDNA insert that was reported to contain the complete coding sequence. The EcoRI fragment containing the insert was inserted into an expression vector constructed in our laboratory. The orientation of the insert in the vector was confirmed by dideoxy sequencing. This expression vector contains two transcription units units that are both driven by the cytomegalovirus immediate early gene promoter. One of the transcription units is used to drive the expression of the inserted FGFR-3 fragment while the second confers resistance to the drug L-histidinol (10). This plasmid was transfected into the ML-20 clone of β-galactosidase expressing MCF-7 cells (11) or the α-18 clone of β-galactosidase and FGF-1 overexpressing MCF-7 cells (2). The latter cell line is tumorigenic in ovariectomized nude mice without estrogen supplementation and forms large aggressive tumors in mice that receive estrogen pellets. The β-galactosidase facilitates the detection of metastases through the use of X-gal staining of excised organs. This would be useful for addressing the separate question as to whether or not FGFR-3 overexpression increased the malignant phenotype of either cell line.

Histidinol resistant colonies were expanded and RNA was extracted from clonal isolates. An RNAse protection assay with an FGFR-3 probe was used to screen 9 α-18 transfected clones and 14 ML-20 transfected clones. A band of 388 nucleotides indicating full protection of the probe was not consistently observed in any of the lanes. However 6/9 of the α-18 transfected clones and 12/14 or the ML-20 transfected clones did show protection of a 130 nucleotide fragment suggesting that the transfected cells were expressing an FGFR-3 mRNA that contained a partial deletion of the area spanned by the probe (Figure 3). This was confirmed by dideoxy sequencing of the FGFR-3 cDNA insert in the expression vector using oligodeoxynucleotide primers that spanned the sequences contained in the riboprobe. The sequencing results indicated a deletion of 380 bp that spanned the juxtamembrane domain and part of the tyrosine kinase domain containing the ATP binding site important for functional kinase activity. This particular cDNA could have potentially arisen though reverse transcription of a splice variant.

If this were the only difference from the published full length sequence, the plasmid would still be

capable of expressing a transmembrane anchored protein containing a complete extracellular domain that would still be useful as a positive control for our studies. However, we did not detect evidence of expression of the protein when we used the antibodies we had generated in a fluorescence activated cell-sorter (FACS) flow cytometric assay with a fluoroscein-labeled anti-rabbit IgG secondary antibody. This caused us to be concerned that other sequence differences that would preclude the generation of a transmembrane anchored protein might also be present in the FGFR-3 cDNA inserted in our expression vector. We therefore prepared oligodeoxynucleotide sequencing primers that spanned the extracellular domain for dideoxy sequencing. We found a large insertion immediatedly before the start of the IIIc exon that encodes the second half of the third IgG loop (12). This again suggests a cDNA that arose from reverse transcription of an alternatively spliced mRNA. We did not complete the sequencing of the inserted sequence but we consider the most likely explanation for the failure to detect cell surface receptor to be due to the presence of an in frame termination codon within the inserted sequence. Since the secretory signal sequence at the amino terminus was maintained, the expressed protein would then be a secreted portion of the extracellular domain rather than being membrane anchored.

The existence of a similar splice variant leading to the production of a secreted extracellular domain has previously been described for FGFR-1 but not for FGFR-3. Despite the absence of the second half of the third IgG domain, the FGFR-1 variant is capable of binding FGF (13,14). The expression of such a variant would therefore be expected to inhibit FGF signal transduction through competition for binding of FGF to the functional transmembrane-anchored receptor tyrosine kinase. We have some preliminary evidence that the expression of the FGFR-3 variant is also capable of inhibiting FGF signal transduction. When compared to control vector transfected cells, the FGF-1 transfected α-18 cells show an increased ability to grow in soft agar under estrogen-depleted conditions. We observed that this ability was partially reversed in clones of α-18 cells that were of transfected with the FGFR-3 expression vector and the extent to which this effect was observed appeared to correlate with the amount of transfected FGFR-3 mRNA expressed (Figures 3 and 4).

C. Development of assays for FGF-7 (a.k.a. keratinocyte growth factor or KGF) and KGF receptor (KGFR) isoform expression in breast cancer cell lines and tumors.

1. Development of a FGF-7 riboprobe for RNAse protection assays.

A 195 bp partial cDNA spanning the first 65 amino acids of FGF-7 was synthesized using reverse transcription-polymerase chain reaction (RT-PCR). This portion was chosen based on its relative lack of homology within this region to corresponding regions of FGFs 1-6. The PCR primers utilized were sense: 5'-ATCCACAAATGGATACTGAC-3' and antisense: 5'-TTATATCCCCTCCTTCCATG-3. The template for RT-PCR was human placental RNA. The reverse transcription and PCR reactions were performed according to the instructions provided by the manufacturer of the RT-PCR kit (Perkin-Elmer). The PCR product was made blunt-ended and ligated into the pGEM7zf+ transcription vector that was linearized with SmaI. The sequence of plasmid inserts from ampicillin resistant colonies arising after transformation was verified by dideoxy sequencing using the T7 P/P primer (Promega). A plasmid having the insert in the orientation: 5'-T7---KGF sense (1-195 bp)---SP6-3' was chosen and plasmid DNA was linearized with EcoRI. A 283 nucleotide ^{32}[P] labeled antisense transcript was prepared and RNAse protection assays were performed as described in (1). RNA was isolated from a number of human breast cancer cell lines and 36 human breast tumor tissues using either guanidium isothiocyanate or RNAzol methods. RNA from the human WI-38 Embryonic lung fibroblast cell line obtained from the ATCC was

used as a positive control. All human breast cancer cell lines were negative for expression of FGF-7 mRNA. However, 35 of 36 breast tumor tissues tested were positive in the assay. A photocopy of a representative experiment with breast tumor RNA is included as Figure 5 in the Appendix.

2. Generation of a riboprobe for the FGFR-2 exon IIIb splice variant.

The high levels of FGF-7 mRNA found in human breast tumors but not in any of the breast cancer cell lines is consistent with what has been observed in a rat prostate tumor model where a dynamic stroma-tumor interaction exists that involves FGF-7 production by stromal cells and expression of the FGFR-2 exon IIIb splice variant in tumor cells. This exon encodes a region of the third immunoglobulin-like domain that confers the FGFR-2 receptor with the specific ability to bind FGF-7 with high affinity (15). In the rat model this paracrine interaction is responsible for maintenance of the tumor in a well differentiated and androgen-dependent state. With tumor progression to an androgen-independent undifferentiated state, there is loss of the expression of the FGFR-2 IIIb splice variant and the ability of the tumor cells to respond to FGF-7. Additionally a second FGFR-2 splice variant, IIIc, is activated to allow response to FGF-2 and the coordinate induction of the FGF-2 ligand itself also occurs. FGFR-1 is also activated in the progressed tumor cells as well (16).

We therefore wished to develop an assay for expression of the FGFR-2 IIIb splice variant to determine if absence of expression in human breast tumors could be correlated with estrogen independence. RT-PCR was also used to generate a 171 bp cDNA corresponding to nucleotides 1345 to 1515 of the FGFR-2 cDNA which spans the region that that contains the IIIb exon. The primers used were sense: 5'-CAAGGTTCTCAAGCACTCGGGGATAAATAGTTCCAA-3', and antisense: 5'-CTTCCAGGCGCTTGCTGTTTTGGCAGG-3'. RNA from the MCF-7 ER+ breast carcinoma cell line was used as the template for the reverse transcription. The conditions for PCR were 94°C, 1 min 60°C, 2 min; 72°C, 1 min; 35 cycles. The PCR product was blunt-ended and ligated to SmaI digested pGEM7zf+ DNA and the inserted sequence was confirmed by dideoxy sequencing. A plasmid with the orientation: 5'-T7---KGFR antisense---SP6-3' was chosen for use. After linearization with Hind III, a 251 nucleotide riboprobe was generated using T7 polymerase. However conditions for RNAse protection could not be successfully established with this probe and an artefactual band was consistently observed.

3. Development of an RT-PCR assay for discriminating FGFR-2 isoforms expressed in breast cancer cell lines and tumors.

As an alternative we developed an RT-PCR assay that allows us to discriminate the IIIb from the IIIc splice variant. This assay is based on the presence of restriction enzyme cleavage sites contained within the amplified PCR product that are specific to each isoform. This assay uses two PCR primers derived from the FGFR-2 sequence which spans the exon IIIb or IIIc borders. The primers therefore hybridize to sequences that are common to both splice variants since they are outside of the region that differs (Figure 6). If the template utilized in the PCR reaction is derived from reverse transcribed FGFR-2 mRNA containing the IIIc exon (Bek mRNA), then a 408 bp sequence is amplified which contains an EcoRV restriction enzyme cleavage site within the it. Subsequent digestion with Eco RV yields two fragments of 320 and 88 bp. If the template utilized in the PCR reaction is derived from reverse transcribed FGFR-2 mRNA that contains the IIIb exon (KGFR mRNA), the amplified DNA is 411 bp in length an contains an AvaI site. Subsequent digestion with this enzyme yields two fragments of 213 and 198 bp that can easily be distinguished on an agarose gel. The PCR primers used were sense: 5'-

GTACCACCTGGATGTTGTG-3' (nucleotides 1147-1165 of the KGFR) and antisense: 5'-GGCTATCTCCAGGTAGTCT-3' (the inverse-complement of nucleotides 1540-1559 of the KGFR). The Perkin-Elmer RT-PCR kit was used. Reverse transcription was performed using one μg of total RNA with random hexamers as primers. The PCR reaction was then carried out according to the manufacturer's instructions.

With this assay we have found that both ER+ and ER- breast cancer cells lines exclusively produce the IIIb isoform. the top left panel of Figure 6 shows the results using the ER+ MCF-7 breast cancer cell line and the ER- MDA MB-468 cell line. In both cases digestion of the PCR-amplified DNA is only observed in the AvaI treated samples. The SW-13 cell line in the figure is an adrenal cortical carcinoma cell line that is used as a control for detection of FGFR-2 IIIc expression. Other ER+ and ER- cell lines exclusively produced the FGFR-2 IIIb KGFR mRNA. The only exception was found in the ER- Hs578T cell line, where the restriction fragments resulting from digestion of the IIIc isoform were observed. RNA from breast tumor tissue was also analyzed using this assay. Specific expression of the exon IIIb variant was found in 10/12 of the tumors examined. There was no indication of expression of the FGFR-2 exon IIIc isoform in any of the breast tumor tissues examined. Figure 6 also contains a representative gel showing such an analysis on two tumor samples.

CONCLUSIONS:

High titer FGFR-3 rabbit antisera have successfully been generated. A suitable number of samples from patients who received hormonal therapy were identified and tumor tissue sections from the majority of these patients were obtained. Progress in the development of the immunohistochemical assay was hampered by the unexpected finding of a new FGFR-3 spice variant which negated our attempts to develop a tumorigenic breast cancer cell line overexpressing FGFR-3 that could be used as a positive control. However, preliminary evidence suggesting the use of secreted FGFR-3 receptor as a potential therapy aimed at inhibiting FGF signal transduction was obtained.

A successful RNAse protection assay for FGF-7 mRNA expression has been developed. The detection of FGF-7 mRNA in human breast tumor tissues but not in any human breast cancer cell lines implies that the expression emanates from the stromal component of the tumor. The probe for KGFR expression was not found to be useful. An RT-PCR assay for KGFR expression in human tumors developed as an alternative indicates that there does not appear a switch in receptor isoform expression which correlates with progression from ER+ to ER-. Instead, our data suggests that a host-tumor interaction exists that involves FGF-7 production by stromal cells and specific expression of the FGFR-2 isoform that binds FGF-7 by both ER+ and ER- tumors. The consequences of this possible interaction remain to be determined, but these results raise the possibility of signal transduction mediated through FGF receptors resulting in growth stimulation of breast tumor cells.

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APPENDIX

six figures attached

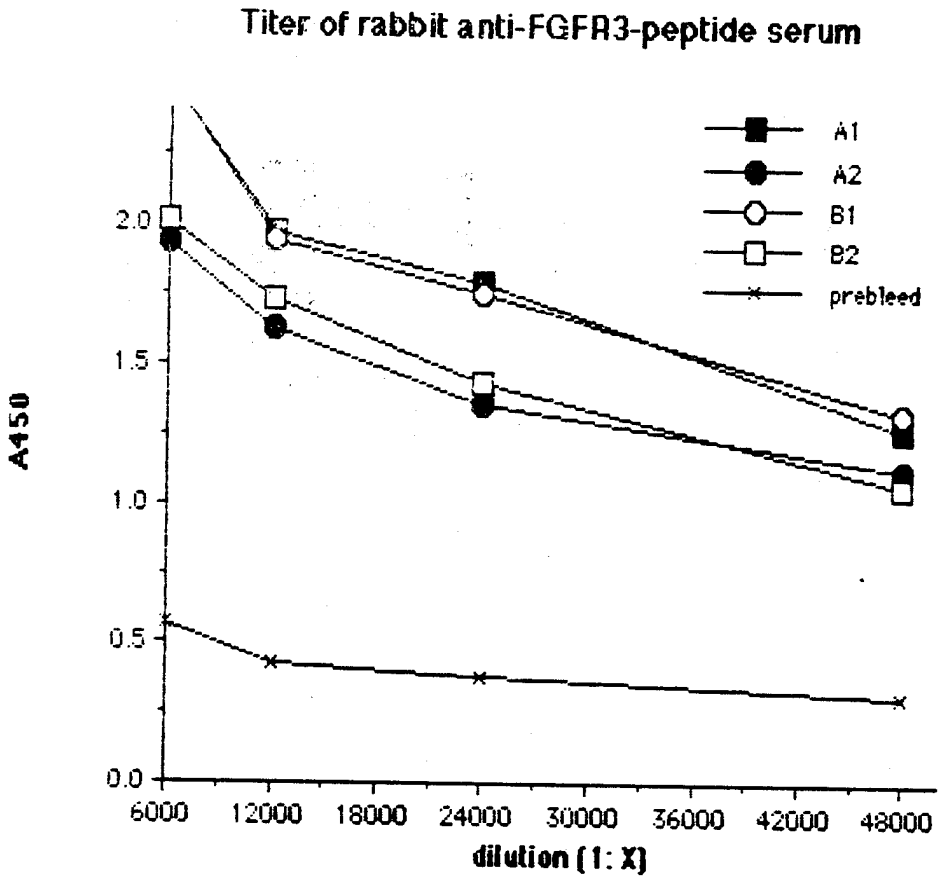
Titer of rabbit anti-FGFR3-peptide serum
A1
A2
B1
B2
prebleed
A450
2.0
1.5
1.0
0.5
0.0
6000
12000
18000
24000
30000
36000
42000
48000
dilution (1: X)

Figure 1

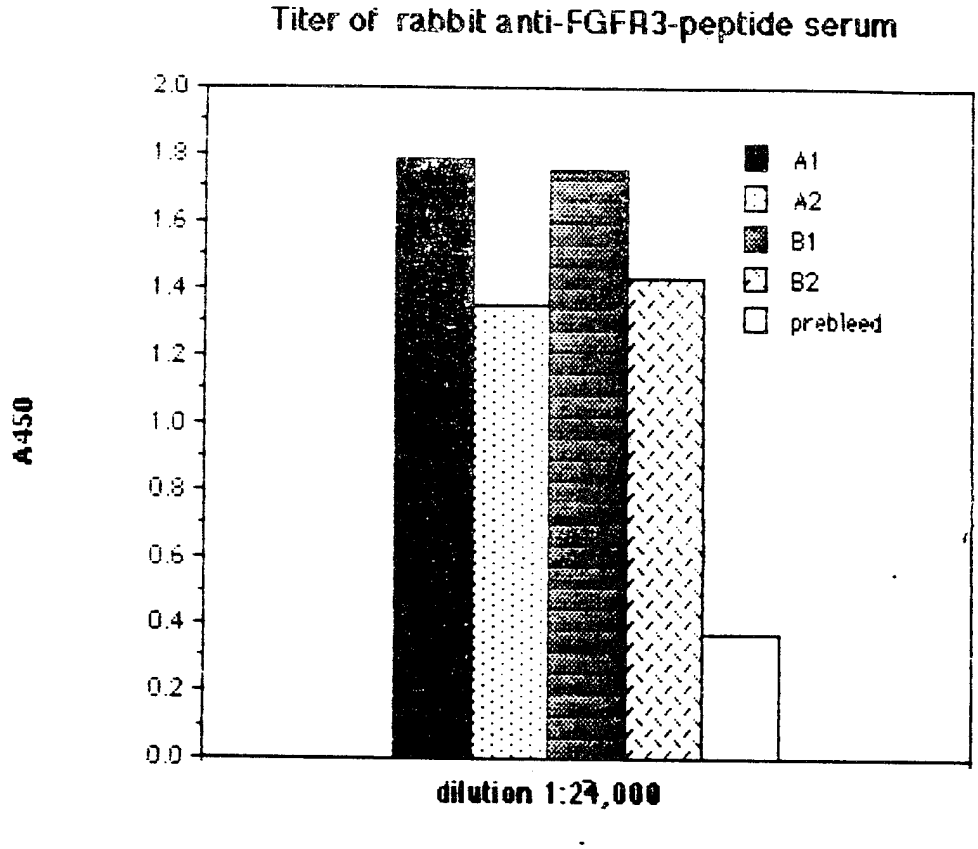
Titer of rabbit anti-FGFR3-peptide serum
A450
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A1
A2
B1
B2
prebleed
dilution 1:24,000

Figure 2

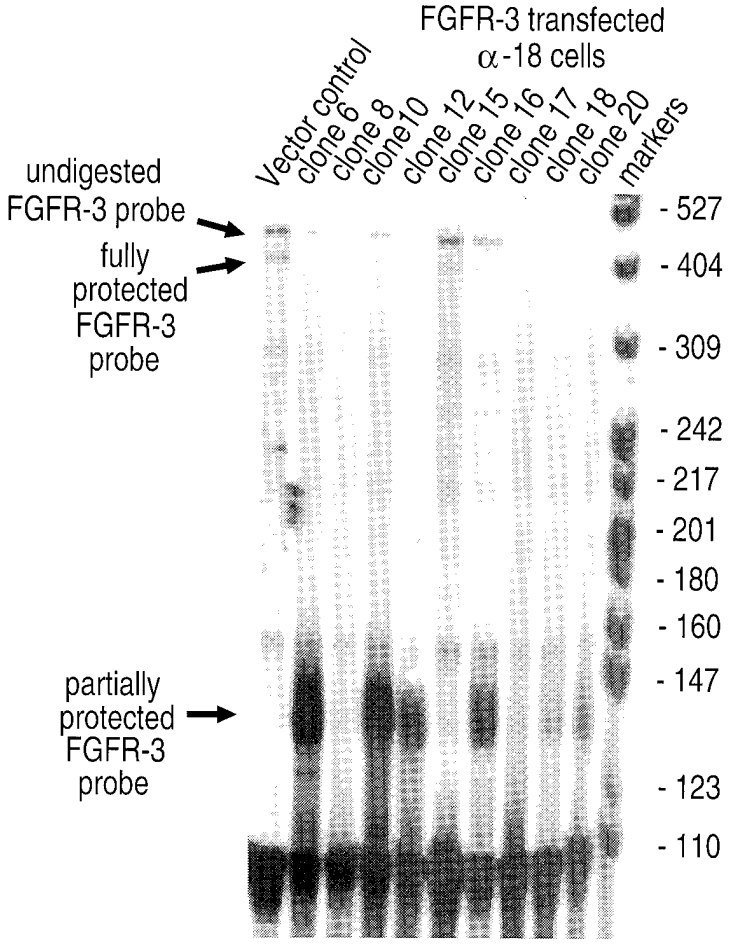

Figure 3. Evidence for expression of aberrant forms of FGFR-3 mRNA in alpha-18 FGF-1 overexpressing MCF-7 cells retransfected with an FGFR-3 expression vector.

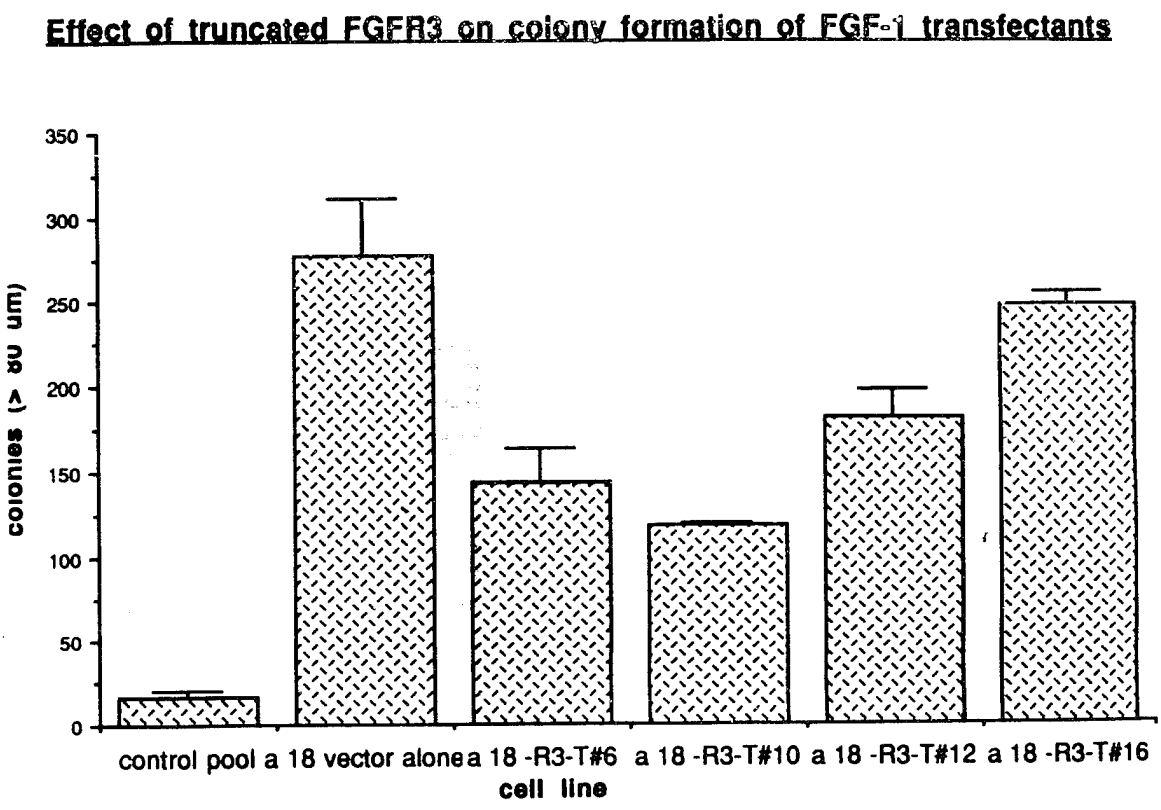

Figure 4. Soft agar cloning assay suggesting a potential inhibitory effect of FGFR-3 extracellular domain secretion on the increased ability of FGF-1 overexpressing MCF-7 cells to form colonies in estrogen-depleted media. 10,000 cells were stripped of estrogen and plated in 0.36% agar in phenol red free media supplemented with 5% charcoal-stripped calf serum. Control pool are MCF-7 cells that do not overexpress FGF-1. A-18 vector alone refers to alpha-18 FGF-1 overexpressing cells transfected with the vector lacking the FGFR-3 cDNA insert. Clones 6,10, 12 and 16 refer to the same FGFR-3 transfected clones used for the RNAse protection assay shown in Figure 3.

Figure 5. Expression of FGF-7 (KGF) mRNA in Human Breast Tumors

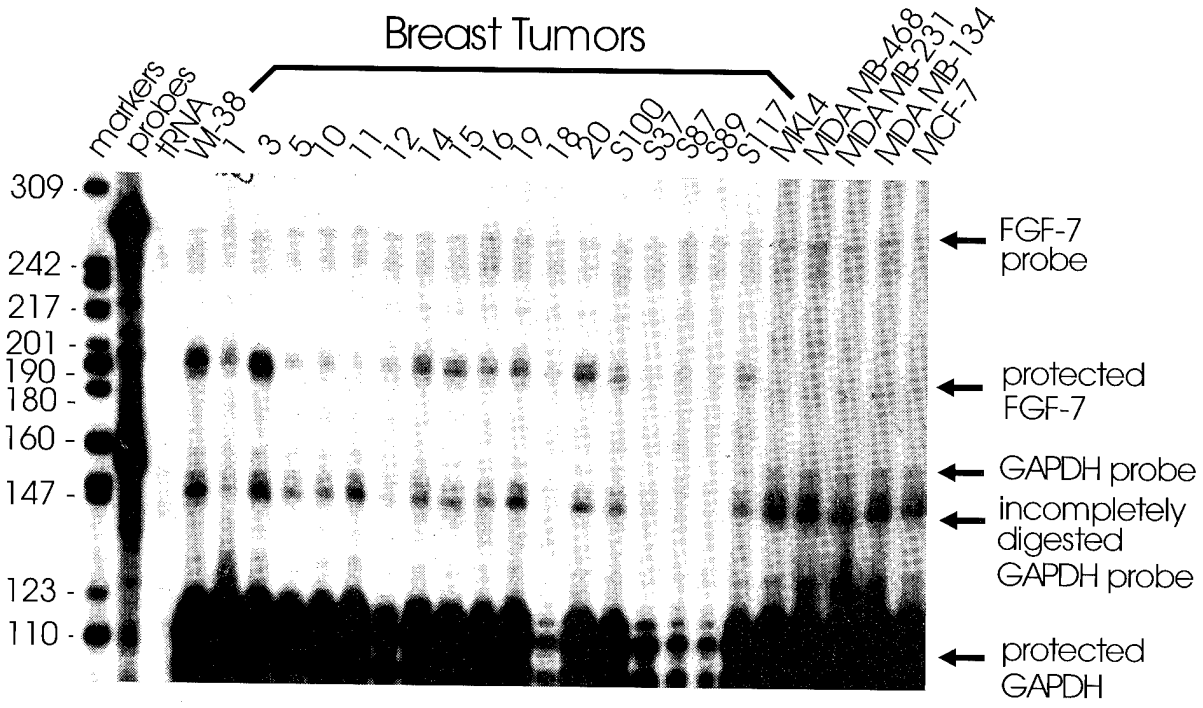

Figure 6. RT- PCR assay for FGFR-2 isoforms in human breast cancer cell lines and tumor tissues